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Identification of the absorbed constituents after oral administration of Yuanhu Zhitong prescription extract and its pharmacokinetic study by rapid resolution liquid chromatography/quadrupole time-of-flight

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ABSTRACT

Yuanhu Zhitong prescription (YZP) is well known for its analgesic effect. However, its multiple bioactive components *in vivo* remain unclear. In this paper, a rapid resolution liquid chromatography/quadrupole time-of-flight (RRLC-ESI-Q/TOF) was employed to identify the bioactive components and partial metabolites after oral administration of YZP extracts. Meanwhile, a RRLC-ESI-Q/TOF method was established and validated for the simultaneous quantification of protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin in rat plasma and applied for their pharmacokinetic research. The results showed that twenty-one bioactive components of YZP were absorbed into the blood circulation and seventeen components were detected in cerebrospinal fluid (CSF). Moreover, the kinetic profiles of six analytes were obtained and the results suggested that the six analytes peaked between 3.5 and 5.0 h and C_{max} ranged from 214.6 to 858.3. The works could provide key information for identification of bioactive constituents and understanding the metabolism as well as pharmacological actions for YZP.

1. Introduction

Traditional Chinese medicine (TCM) has been used in China for thousands of years and has played an indispensable role in prevention and treatment of diseases, especially for complicated and chronic conditions [1,2]. The herbs used in TCM are usually a complex prescription containing hundreds or even thousands of chemically distinct components in each ingredient, which become a huge challenge to determine which one is responsible for a certain therapeutic effect [3]. Most of TCM formulations are taken orally, and some constituents persist at detectable levels, along with their metabolites, after digestion and absorption. Therefore, it is necessary to trace the constituents of TCM formulation *in vivo* and evaluate their pharmacokinetic profiles which can gain more in-depth insights into the active components and the therapeutic mechanisms for TCM formulation, especially for the drugs working in the brain system [4]. Recently, a RRLC-ESI-Q/TOF method has been reported to boast a higher sensitivity than other methods for identifying known and unknown compounds in complex matrices [5,6]. Thus, it could be a valuable analytical technique for identifying active constituents and evaluating their pharmacokinetic properties of TCM herbs *in vivo*.

Yuanhu Zhitong prescription (YZP) consists of *Radix Angelicae dahuricae* and *Rhizoma corydalis* (processed with vinegar) and is widely used for the treatment of gastralgia, costalgia, headache, and dysmenorrhea caused by qi stagnancy and blood stasis, as recorded in the Chinese Pharmacopoeia [7]. Previous studies indicated that YZP exhibited therapeutic effects through a wide variety of actions, including anti-nociceptive [8], anti-inflammatory [9], anxiolytic [10], spasmolysis [11], and vasorelaxation [12]. Furthermore, *Radix Angelica dahuricae* extracts produced synergistic

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actions on the analgesic effect of *Rhizoma corydalis* alkaloid by increasing plasma concentrations of DL-tetrahydropalmatine [13]. Alkaloids in *Rhizoma corydalis* and coumarins in *Radix Angelicae dahuricae*, had been identified as the active components; moreover, alkaloids, such as DL-tetrahydropalmatine, protopine and berberine, and coumarins, such as imperatorin and isoimperatorin, had been studied to determine their pharmacological effects [14–18]. Recently, we had developed a system to detect 17 constituents of YZP tablet using a RRLC technique coupled with a triple quadrupole mass spectrometry (RRLC-QQQ) [19].

With the recent advances in new mass analyzers, hyphenated technologies and data acquisition software, the qualitative and quantitative analysis of bioactive compounds in TCM herb *in vivo* are more easy to carry out. In addition, this could be applied to pharmacokinetic study of YZP based on quantitative analysis that would identify potentially effective constituents in this herbal formula. These studies might play a very significant role in certain therapeutic effects and warrant further study.

2. Experiments

2.1. Chemicals and materials

HPLC-grade acetonitrile, formic acid, and methanol were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Billerica, MA, USA). The standards of imperatorin, isoimperatorin, psoralen, protopine, and DL-tetrahydropalmatine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Byakangelicin, byakangelicol, and coptisine were acquired from Chengdu Herbpurify Co. (Chengdu, China). Xanthotoxin, oxypeucedanin, and tetrahydroberberine were purchased from Shanghai Winherb Co. (Shanghai, China). Bergapten was obtained from Shanghai Baiyi Biotech (Shanghai, China). Corydaline was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). α-Allocryptopine was supplied by Shenzhen Meihe Biotech (Shenzhen, China). The purities of all standards were no less than 98% and suitable for RRLC-ESI-Q-TOF analysis.

Angelica dahurica (Fisch. ex Hoffm.) Benth. et Hook. f. and *Corydalis yanhusuo* W. T. Wang were obtained from the Anguo Medicinal Material Market (Hebei, China) in February 2011, and the drugs were identified by Pharmacist Xirong He (research assistant at the China Academy of Chinese Medical Sciences). Voucher specimens were deposited in the Institute of Chinese Materia Medica (China Academy of Chinese Medical Sciences).

2.2. Instrument and RRLC-ESI-Q/TOF conditions

Chromatographic experiments were performed on an Agilent 1200 Rapid Resolution Liquid Chromatography System (Agilent Crop., Santa Clara, CA, USA) equipped with a quaternary pump, an online vacuum degasser, an autosampler and an automatic thermostatic column oven. The separation was carried out on Poroshell DB C_{18} column (3.0 mm × 150 mm, 2.7 μm, Agilent) at 35 °C with a flow rate of 0.4 mL/min and injection volume was 5 μL. Mobile phase was a mixture of 0.1% formic acid–water (A) and acetonitrile (B). The gradient program of mobile phase was carried out as follows: 0–10 min, 20–30% B; 10–25 min, 30–60% B; 25–35 min, 60–100% B; 35–40 min, 100% B.

MS detection was conducted on an Agilent 6520 quadrupole time-of-flight mass spectrometer equipped with an electrospray ionization source (ESI). Ionization was performed in the positive electrospray mode. On the basis of the best response for most compounds, the final parameters were as follows: fragmentor (150 V), Vcap (3500 V), nebulizer (30 psi), drying gas (N_2, 10 L/min, 350 °C). The TOF-MS was calibrated daily, according to the manufacturer's recommendations. The testing mass range was set from m/z 50 to 1000 with a scanning rate of 2 s^{-1}. Reference masses at m/z 121.05087 (purine, 1.125×10^{-6} mol/L, Agilent Corp.) and m/z 922.00980 (hexakis (1H, 1H, 3H-tetrafluoropropoxy)-phosphazine, 5×10^{-6} mol/L, Agilent Corp.) were continually introduced, along with the LC stream for accurate mass calibration. The collision energy for each compound varied according to this formula: [5 × (mass/100)] + 5. For example, the collision energy for an ion with nominal m/z of 300 would be 20 V.

2.3. Preparation of YZP extract and the solutions for analysis in vitro

The procedures of YZP extract preparation were as follows: Herbs were ground into powders, respectively. Based on the optimum extraction conditions in the previous research [12], a total of 400 g of *Corydalis yanhusuo* and 200 g of *Radix Angelica dahuricae* were macerated with 2.4 L of 70% ethanol for 2 h, and then extracted twice under thermal reflux for 2 h. The extracts were filtrated out by running through absorbent cotton inserted in a funnel. The two extracts were combined and evaporated to less than 200 mL in a rotary evaporator R-210 (Buchi Ltd., Labortechnik AG, Switzerland) at 50 °C under reduced pressure. Subsequently, the concentrated extracts were freeze-dried using a freeze dryer (TF-FD-1; Tianfeng Instrument Co., Shanghai, China) at −30 °C, and then pressed into powder with a 60-mesh sieve.

2.4. Preparation of standards, calibration standard and quality control (QC) samples

Standard stock solutions for 15 components (protopine, α-allocryptopine, DL-tetrahydropalmatine, coptisine, berberine, tetrahydroberberine, corydaline, bergapten, byakangelicin, xanthotoxin, oxypeucedanin, byakangelicol, imperatorin, psoralen, and isoimperatorin) were prepared respectively by weighing the required amounts into volumetric flasks and dissolving in 75% methanol.

Calibration curve stock solutions of six analytes including protopine, α-allocryptopine, DL-tetrahydropalmatine, tetrahyberberine, corydaline and byakangelicin were prepared in 75% methanol at 1 mg/mL. Prior to use, all stock solutions were diluted with 75% methanol to obtain the combined working solution with the concentration of 10 μg/mL for the six analytes. The combined solution was further diluted with methanol in formation of the following standard solutions, with the concentration of 50, 100, 250, 500, 1000, 2000 and 5000 ng/mL respectively for five standards including protopine, α-allocryptopine, DL-tetrahydropalmatine, tetrahyberberine, corydaline and 15, 30, 75, 150, 300, 600 and 1500 ng/mL for byakangelicin. All the mixed standard solutions were stored at 4 °C.

100 μL combined working solution was evaporated to dryness at 37 °C under the stream of nitrogen, to which 200 μL blank rat plasma were spiked sequentially, and were vigorously vortex mixed for 30 s. For method validation, QC samples were prepared independently according to the same sample preparing protocol, with a spiked concentration of 250 ng/mL for protopine, α-allocryptopine, DL-tetrahydropalmatine, tetrahyberberine, corydaline and 75 ng/mL for byakangelicin.

2.5. Preparation of plasma and CSF samples

The rat plasma samples were prepared by liquid–liquid extraction with ethyl acetate. A 200 μL aliquot of ethyl acetate was added to a clean tube containing 100 μL of plasma sample, thoroughly mixed by vortex for 3 min, then centrifuged at 2000 × *g* for 15 min

at 4 °C. The supernatant was transferred to a clean Eppendorf tube. This procedure was repeated twice, after which the supernatant was evaporated to dryness at 37 °C under a stream of nitrogen. The residue was dissolved in 100 μL of 75% methanol. Subsequently, the tubes were vigorously vortex-mixed for 2 min, and the supernatant collected by centrifugation at 12,000 × *g* for 15 min was directly injected into the RRLC-ESI-Q/TOF system for further analysis. Preparation of the CSF samples was the same as that of blood samples.

2.6. Method validation

Calibration curves were established first by spiking blank samples with appropriate amounts of six standards including protopine, α-allocryptopine, tetrahydropalmatine, tetrahyberberine, corydaline and byakangelicin using six different concentrations described above for plasma samples; then calibration curves were constructed by plotting the peak area *versus* the spiked concentrations of least square linear regression analysis with a weighting factor of $1/x^2$. Deviations of these back-calculated concentrations from calibration standard samples were set within ±15% of nominal concentrations (±20% for the lower limit of quantitation). The concentrations of QC samples and test samples were calculated from the linear regression equation of the peak area ratio against concentrations.

Accuracy and intra/inter-day precision were evaluated by assessing three batches on separate days, each containing five replicates of QC samples. Concentrations were calculated with calibration curves obtained daily. Relative standard deviation (RSD) selected for intra/inter-day precision assessment should not exceed 15%.

The lower limit of quantitation (LLOQ) of the assay was assessed as the lowest concentration of the calibration curve that can be quantitatively determined with acceptable precision less than 20% and accuracy within ±20%. The LLOQ was established based on six replicates on three consecutive days.

The matrix effect (ME) was expressed as the ion suppression/enhancement on the ionization of analyte. The matrix effect of the analytes was investigated by comparing the corresponding peak areas of the post-extraction spiked samples from six different lots of blank plasma to those of the QC working solutions reconstituted in mobile phase. Experiments were performed using QC samples in six replicates. The extraction recoveries were calculated by comparing the peak areas of blank plasma samples spiked with the analytes pre-extraction with those of the post-extraction spiked samples at the same concentrations. Experiments were performed using QC samples in six replicates.

The stability in rat plasma was investigated by comparing the measured concentrations of QC samples in six replicates with the spiked concentrations under the following two storage conditions: (1) short-term temperature stability at room temperature for 6 h; (2) post-preparative stability at 10 °C for 24 h. The analytes was considered stable when the percent deviation was within ± 15%.

2.7. Pharmacokinetic study

Male Sprague-Dawley rats (230 ± 20 g) were obtained from the Experimental Animal Center of Peking University Health Science Center (Beijing, China). Animals were housed in a breeding room with temperature of 24 ± 2 °C, humidity of 60 ± 5%, and 12/12 h dark-light cycle. Tap water and food were supplied *ad libitum*, but animals were fasted (with free access to water) for 12 h prior to the experiments. The animal welfare and experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council of the USA, 1996) and related ethical regulations of China Academy of Chinese Medical Sciences. The protocols were approved by the Animal Ethics Committee of Laboratory Animals of the Institute of Laboratory Animal Resources of Beijing (Beijing, China).

Rats were orally administered YZP extract at a dose of 2.35 g/kg (equivalent to crude drug 24 g/kg) that the administration doses of six individual compounds including protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin were 4.54, 5.82, 6.98, 14.05, 2.66 and 1.78 mg/kg, respectively. At the same time, three standard compounds at a dose of 1.0 mg/kg including protopine, tetrahydropalmatine and corydaline, individually. At 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 12 h after YZP administration, blood samples from the common carotid artery were collected in polythene tubes with anticoagulant heparin sodium (100 unit/mL). Then, the blood samples were centrifuged (TGL-16G; Shanghai Precision Instruments Co., Ltd., Shanghai, China) at 2000 × *g* for 15 min at 4 °C. The supernatants were stored at −20 °C. CSF samples were collected from cerebellum medulla oblongata cistern *via* percutaneous puncture at 1 h post-YZP administration. Major pharmacokinetic parameters, including C_{max}, T_{max} and AUC_{0-t}, were calculated by the Drug and Statistics (DAS) 3.0 software (edited by Chinese Mathematical Pharmacology Society).

3. Results and discussion

3.1. Optimization of RRLC-ESI-Q/TOF condition

To obtain reliable chromatographic results and appropriate ionization, four mobile phase systems of acetonitrile–water, methanol–water, acetonitrile-acid aqueous solution and methanol-acid aqueous solution were tested and compared. The results suggested that acetonitrile-acid aqueous solution was superior to the others. Meanwhile, formic acid was added into the mobile phase to improve the peak shape and restrain the peak tailing, as well as increase ion response for alkaloids and coumarins. This finding was confirmed by the optimal solvent systems consisting of a mixture of 0.1% formic acid–water (A) and acetonitrile (B). The absorbed constituents of YZP belonged to different chemical families and displayed distinct polarities in complex biological matrix; thus, gradient elution was employed to obtain effective separation, guarantee high ionization, and minimize ion suppression. For the ESI-Q/TOF conditions, ESI was determined in both negative and positive ion modes, and the results indicated that the conditions were obtained from the ESI positive ionization mode which could achieve a higher response and provide more information for all compounds than the ESI negative mode in MS spectra.

3.2. Optimization of sample preparation

Various methods of sample preparation were tested to select an efficient extraction method for obtaining superior recovery of target compounds and reducing matrix effects from plasma samples. The methods included protein precipitation with acetonitrile or methanol and liquid–liquid extraction. Direct protein precipitation with acetonitrile or methanol could only measure a few constituents possibly because of poor recovery and strong ion suppression. Finally, liquid–liquid extraction was adopted because this technique could increase the signal intensity. Different solvent including ethyl acetate, chloroform and diethyl ether were investigated and ethyl acetate was selected as extraction solvent due to its high extraction efficiency and the low interference, which was in accord with the previous report [20]. Subsequently, different volumes (100, 200 and 300 μL) of the solvent and extraction times (1, 2 and 3) were investigated, respectively. The results suggested that

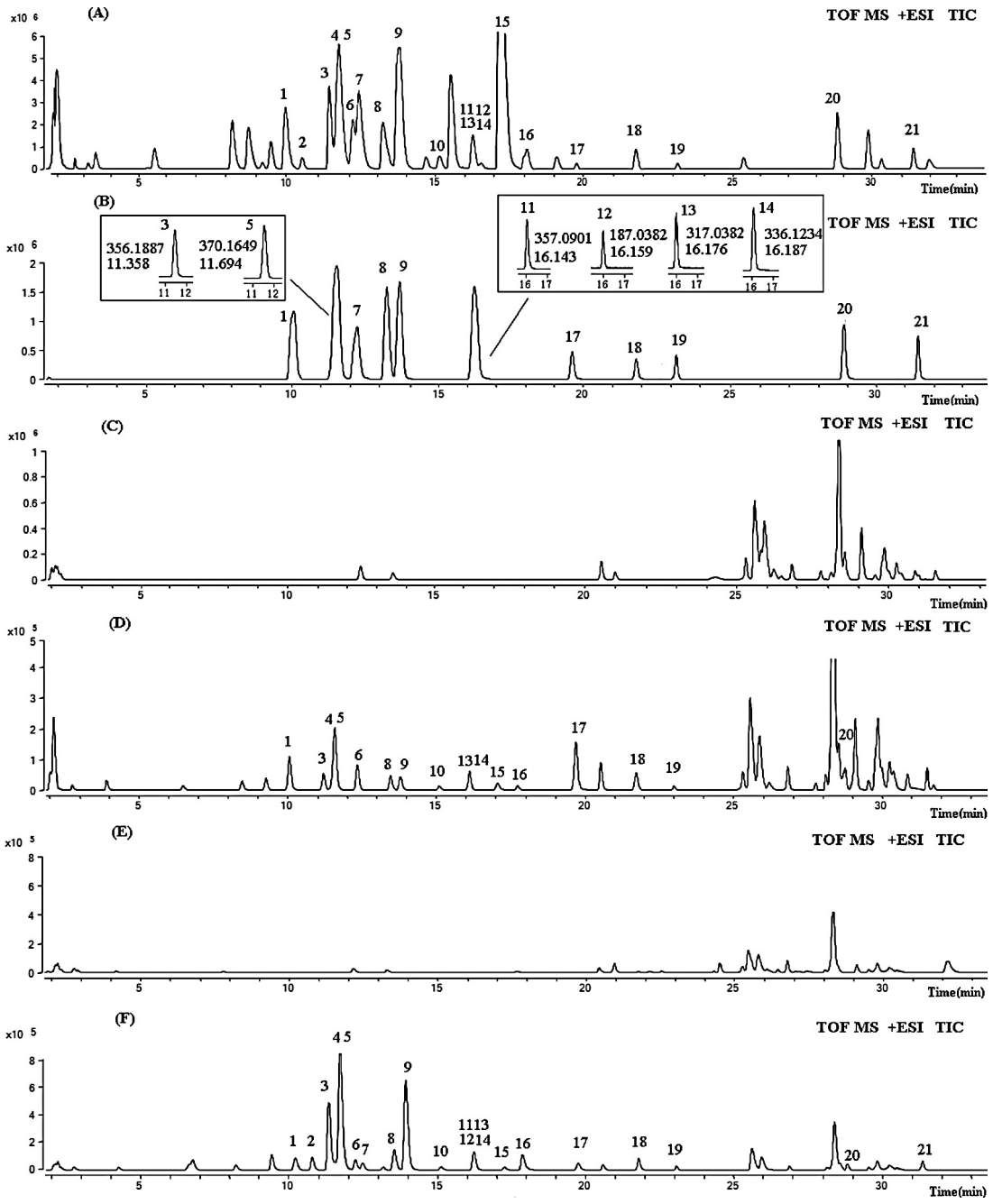

Fig. 1. RRLC-ESI-Q/TOF chromatograms (TCC) of (A) the solution of YZP, (B) 15 mixture references solution, (C) blank CSF samples, (D) drug CSF samples after oral administration of YZP extract, (E) blank plasma samples, and (F) drug plasma samples after oral administration of YZP extract from positive ion mode. Peaks: 1, protopine; 3, tetrahydropalmatine; 5, α-allocryptopine; 7, coptisine; 8, tetrahydroberberine; 9, corydaline; 11, byakangelicin; 12, psoralen; 13, byakangelicol; 14, berberine; 17, xanthotoxin; 18, bergapten; 19, oxypeucedanin; 20, imperatorin; and 21, isoimperatorin.

200 μL aliquot of ethyl acetate adding to 100 μL of plasma sample and could be extracted completely within two times.

3.3. Screening and analysis of prototype constituents and metabolites in rat plasma and CSF

To clarify the active constituents responsible for pharmacological actions, it is necessary to first acquire chemical profiles of TCM herb medicine *in vivo*. Therefore, in this study, the RRLC-ESI-Q/TOF technique was developed to identify the multiple components in plasma and CSF after oral administration of YZP extracts to Sprague-Dawley rats. RRLC chromatograms of fifteen mixture references solution, the solution of YZP, blank samples (plasma and CSF), and drug samples (plasma and CSF) after oral administration of YZP extract were shown in Fig. 1. The retention time (t_R), MS data, and fragments of absorbed constituents were listed in Table 1, and the chemical structures of standards compounds were shown in Fig. 2.

The constituents in rat plasma and CSF after oral administration of YZP extracts were well separated and identified by their retention time and mass spectra. By comparing the chromatogram of YZP extract with that of the standards, twenty-one peaks were identified in the plasma and seventeen peaks in the CSF (Table 2). Among the twenty-one absorbable compounds in plasma, fifteen constituents (peaks 1, 3, 5, 7–9, 11–14, and 17–21) were identified as protopine, DL-tetrahydropalmatine, α-allocryptopine, coptisine, tetrahydroberberine, corydaline,

Table 1
MS data of ESI-MS spectra and identification of the YZP constituents.

Peak no.	RT (min)	Experimental mass (*m*/*z*)	Calculated mass (*m*/*z*)	Formula	MS/MS (*m*/*z*)	Source	Identified compound
1	10.09	354.1331	354.1336	$C_{20}H_{19}O_5N$	206.0804[RDA-tetrahydroisoquinoline] 149.0596[RDA-benzene-ring]	Plasma, CSF	Protopine
2	10.79	356.1863			192.1003,177.0789	Plasma	Unknown
3	11.36	356.1887	356.1856	$C_{21}H_{25}O_4N$	192.1005[RDA-tetrahydroisoquinoline] 177.0789 [RDA-benzene-ring]	Plasma, CSF	Tetrahydropalmatine
4	11.65	356.1861			192.1003,177.0784	Plasma, CSF	Unknown
5	11.69	370.1678	370.1649	$C_{21}H_{23}NO_5$	352.0931[M−H_2O]$^+$ 206.0795[RDA-tetrahydroisoquinoline] 165.0914[RDA-benzene-ring]	Plasma, CSF	α-Allocryptopine
6	12.24	324.1194			176.0694,149.0589	Plasma, CSF	Unknown
7	12.48	320.0910	320.0917	$C_{19}H_{15}NO_4$	292.0922[M−CO]$^+$ 262.0866[M−2CO-2H]$^+$	Plasma	Coptisine
8	13.59	340.1575	340.1543	$C_{20}H_{21}O_4N$	176.0706[RDA-benzene-ring]	Plasma, CSF	Tetrahydroberberine
9	13.71	370.2010	370.2013	$C_{22}H_{27}NO_4$	207.1205[RDA-tetrahydroisoquinoline] 191.0897[RDA-benzene-ring]	Plasma, CSF	Corydaline
10	15.32	305.1021			203.0331	Plasma, CSF	Unknown
11	16.14	357.0901	335.1125	$C_{17}O_{18}O_7$	231.0156[M−H_2O-C_5H_8O]$^+$ 203.0348[M−H_2O-C_5H_8O-CO]$^+$	Plasma, CSF	Byakangelicin
12	16.16	187.0384	187.0382	$C_{11}H_6O_3$	159.1311[M−CO]$^+$ 144.0484[M−$COCH_3$]$^+$ 131.0532 [M−2CO]$^+$	Plasma	Psoralen
13	16.18	317.1043	317.102	$C_{17}H_{16}O_6$	287.0879[M−CH_2O]$^+$ 203.0338[M−CH_2O-C_5H_8O]$^+$ 175.0402[M−CH_2O-C_5H_8O-CO]$^+$	Plasma, CSF	Byakangelicol
14	16.19	336.1192	336.1230	$C_{20}H_{18}NO_4$	321.0995[M−CH_3]$^+$ 292.0944[M−CH_3-H-CO]$^+$	Plasma, CSF	Berberine
15	17.18	366.1706	366.1700	$C_{22}H_{24}NO_4$	351.1445[M−CH_3]$^+$ 336.1182[M−$2CH_3$]$^+$ 322.1420[M−CH_3-H-CO]$^+$ 308.1298[M−$2CH_3$-CO]$^+$	Plasma, CSF	Dehydrocorydaline
16	17.89	352.1536	352.1543	$C_{21}H_{22}NO_4$	337.1272[M−CH_3]$^+$ 322.1078[M−$2CH_3$]$^+$ 308.1268[M−CH_3-H-CO]$^+$	Plasma, CSF	Palmatine
17	19.90	217.0508	217.0495	$C_{12}H_8O_4$	202.0257[M−CH_3]$^+$ 161.0598[M−2CO]$^+$	Plasma, CSF	Xanthotoxin
18	21.90	217.0504	217.0495	$C_{12}H_8O_4$	202.0256[M−CH_3]$^+$ 174.0322[M−CH_3-CO]$^+$	Plasma, CSF	Bergapten
19	23.02	287.0877	287.0914	$C_{16}H_{14}O_5$	203.0331[M−C_5H_8O]$^+$	Plasma, CSF	Oxypeucedanin
20	28.91	271.0958	271.0965	$C_{16}H_{14}O_4$	203.0314[M−C_5H_8]$^+$	Plasma, CSF	Imperatorin
21	31.47	271.1097	271.0965	$C_{16}H_{14}O_4$	203.0336[M−C_5H_8]$^+$ 159.0388[M−C_5H_8-CO_2]$^+$ 147.0438[M−C_5H_8-2CO]$^+$	Plasma	Isoimperatorin

The ions at *m*/*z* 357.0901 were [M+Na]$^+$.

byakangelicin, byakangelicol, berberine, psoralen, xanthotoxin, bergapten, oxypeucedanin, imperatorin, and isoimperatorin by comparing with the chromatographic-mass spectrometric analysis of standard compounds. Peak 15 and 16 showed a quasi-molecular ion at *m*/*z* 366.1671 and *m*/*z* 352.1500 in MS spectra respectively. Meanwhile, *m*/*z* 351.1445 [M−CH_3]$^+$, *m*/*z* 322.1420[M−CH_3-H-CO]$^+$ ions could be detected in MS/MS spectra of peak 15. Similarly, *m*/*z* 337.1272 [M−CH_3]$^+$, *m*/*z* 308.1248 [M−CH_3-H-CO]$^+$ ions could be measured in MS/MS spectra of peak 16. The same fragment ions of both compounds at *m*/*z* 15 and *m*/*z* 42 were characteristic

Table 2
Summary of the metabolites of tetrahydropalmatine, protopine and corydaline in rat plasma.

No.	RT (min)	[M+H]$^+$	Metabolic reaction	Formula	Source	Parent compound
M1-1	3.83	328.1539	2× demethylation	$C_{19}H_{21}NO_4$	Plasma	Tetrahydropalmatine
M1-2	6.78	358.1646	Demethylation, hydroxylation	$C_{20}H_{23}NO_5$	Plasma	Tetrahydropalmatine
M1-3	8.01	372.1800	Hydroxylation	$C_{21}H_{25}NO_5$	Plasma, CSF	Tetrahydropalmatine
M1-4	8.81	372.1800	Hydroxylation	$C_{21}H_{25}NO_5$	Plasma, CSF	Tetrahydropalmatine
M1-5	9.21	342.1691	Demethylation	$C_{20}H_{23}NO_4$	Plasma, CSF	Tetrahydropalmatine
M1-6	10.01	342.1695	Demethylation	$C_{20}H_{23}NO_4$	Plasma, CSF	Tetrahydropalmatine
M2-1	4.79	342.1333	Demethylation, hydrogenation	$C_{19}H_{19}NO_5$	Plasma	Protopine
M2-2	5.92	342.1365	Demethylation, hydrogenation	$C_{19}H_{19}NO_5$	Plasma	Protopine
M3-1	4.90	400.1747	Hydroxylation, ketone formation	$C_{22}H_{25}NO_6$	Plasma	Corydaline
M3-2	5.53	400.1753	Hydroxylation, ketone formation	$C_{22}H_{25}NO_6$	Plasma	Corydaline
M3-3	7.63	400.1756	Hydroxylation, ketone formation	$C_{22}H_{25}NO_6$	Plasma	Corydaline
M3-4	10.01	368.1854	Hydroxylation, dehydration	$C_{22}H_{27}NO_5$	Plasma	Corydaline
M3-5	11.37	368.1853	Hydroxylation, dehydration	$C_{22}H_{27}NO_5$	Plasma	Corydaline

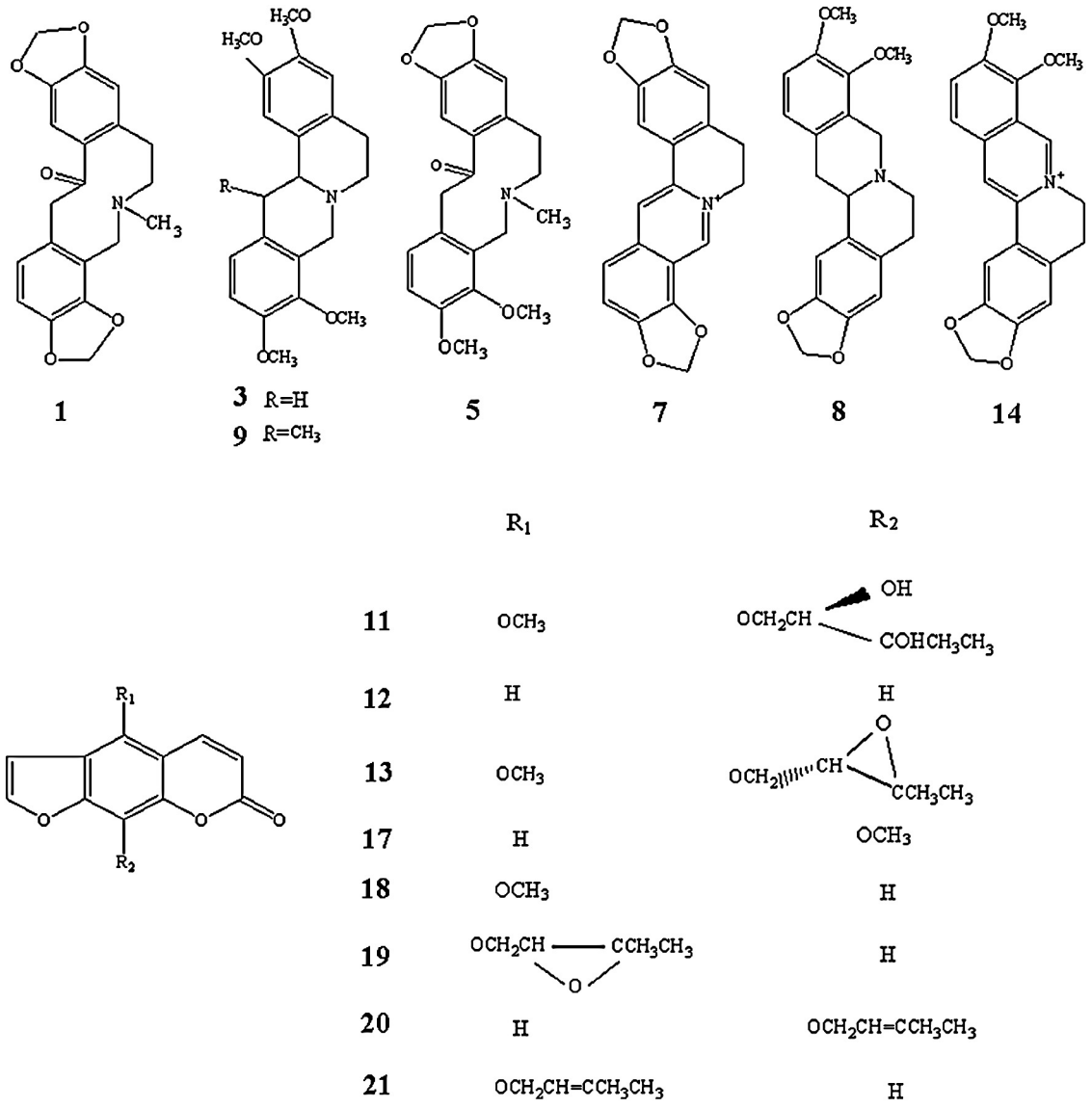

Fig. 2. Chemical structures of standard compounds. 1, protopine; 3, tetrahydropalmatine; 5, α-allocryptopine; 7, coptisine; 8, tetrahydroberberine; 9, corydaline; 11, byakangelicin; 12, psoralen; 13, byakangelicol; 14, berberine; 17, xanthotoxin; 18, bergapten; 19, oxypeucedanin; 20, imperatorin; 21, isoimperatorin.

ions to identify as dehydrocorydaline and palmatine according to previous data [21,22]. However, these two components could not be confirmed due to the lack of related standards. In addition, four compounds (peaks 2, 4, 6, and 10) were unknown and require further study. Importantly, seventeen compounds (peaks 1, 3–6, 8–11, and 13–20) were detected in CSF, suggesting that they had passed through the blood-brain barrier (BBB), which might explain the central analgesia effect of YZP.

The plasma metabolites were very helpful for revealing the bioactive chemical basis of YZP. Up to now, the literature data on the metabolism of YZP was still limited. Meanwhile, because of complex chemical system, it was very difficult to identify the metabolites after the administration of the extracts of TCM formulation. Thus, it was necessary to carry out the metabolism after the administration of single component and TCM formulation in order to obtain the effective and reliable results. In the current studies, three constituents including protopine, tetrahydropalmatine and corydaline which existed plentifully in YZP, were orally administrated individually in order to conclude the structures of the partial metabolites of YZP. The detailed information of these metabolites, including the retention times, proposed elemental compositions, and the molecule ions were listed in Fig. 3 and Table 2. After oral administration of single compound, the results suggested that tetrahydropalmatine was metabolized into six metabolites by 2× demethylation, demethylation and hydroxylation, hydroxylation and demethylation, respectively. Protopine had occurred a reaction of demethylation and hydrogenation into two metabolites. Meanwhile five metabolites from corydaline were produced by hydroxylation and ketone formation, hydroxylation and dehydration, respectively. At the same time, all those metabolites could be detected in the rat plasma samples after oral administration of YZP extracts.

3.4. Method validation

3.4.1. Linearity and LOQ

Regression equation, linear ranges, correlation of coefficients were shown in Table 3. All analytes' calibrations exhibited excellent linearity with coefficients (r) higher than 0.992. As seen in Table 4, LLOQ of protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin were 51.40, 48.02, 49.32, 44.91 34.01 and 9.29 ng/mL respectively, with accuracy (recovery)

Table 3
Regression data regression of the analytes determined.

Analytes	Linear equation[a]	Correlation coefficient (r)	Linear range (ng/mL)
Protopine	$y = 14256x + 548{,}370$	0.9921	50–5000
α-Allocryptopine	$y = 23596x + 748{,}610$	0.9932	50–5000
Tetrahydropalmatine	$y = 18488x + 360{,}140$	0.9992	50–5000
Tetrahyberberine	$y = 17212x + 499{,}280$	0.9962	50–5000
Corydaline	$y = 26827x + 684{,}050$	0.9986	50–5000
Byakangelicin	$y = 23596x + 748{,}610$	0.9928	15–1500

[a] The regressive equations are presented as $y = ax + b$. y is the peak area, x is the concentration of compound.

Table 4
LLOQs of the analytes in the rat plasma ($n = 6$).

Analytes	Measure conc. (ng/mL)	Precision (% RSD)	Accuracy (% recovery)
Protopine	51.40 ± 3.99	7.78	102.80
α-Allocryptopine	48.02 ± 2.53	5.26	99.81
Tetrahydropalmatine	49.32 ± 2.81	5.71	96.10
Tetrahyberberine	44.91 ± 2.25	5.01	94.31
Corydaline	34.01 ± 3.64	7.76	95.25
Byakangelicin	9.29 ± 0.76	8.19	85.60

RSD: Relative standard deviation.

Table 5
The precisions, repeatability, matrix effects, recovery and stability of the analytes in the rat plasma ($n = 6$).

Analytes	Spiked conc. (ng/mL)	Precision		Matrix effects (%) ($n = 6$)	Recovery (%) ($n = 6$)	Stability (RSD %, $n = 6$)	
		Intra-run (RSD %)	Inter-run (RSD %)			At room temperature for 6 h	At 10 °C in the autosampler for 24 h
Protopine	250	4.2	7.1	107.3 ± 2.9	93.8 ± 7.6	6.14	7.71
α-Allocryptopine	250	7.0	5.8	107.8 ± 3.0	85.2 ± 8.8	4.79	6.70
Tetrahydropalmatine	250	3.9	6.3	105.2 ± 2.1	92.9 ± 8.2	7.12	4.54
Tetrahyberberine	250	6.7	8.6	107.2 ± 1.5	89.3 ± 7.9	8.23	6.72
Corydaline	250	3.5	7.2	107.7 ± 3.3	88.1 ± 8.9	5.35	8.63
Byakangelicin	75	4.8	4.5	98.9 ± 4.9	90.8 ± 6.7	5.76	8.13

between 85.60% and 102.80% and precision (RSD) less than 8.13%, which was appropriate for quantification of six analytes in the pharmacokinetic study.

3.4.2. Extraction recovery and matrix effect

A summary of matrix effect and extraction recovery was shown in Table 5. The results of matrix effect for protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin were 107.3 ± 2.9%, 107.8 ± 3.0%, 105.2 ± 2.1%, 107.2 ± 1.5%, 107.7 ± 3.3% and 98.9 ± 4.9% respectively, suggesting that no significant suppression or enhancement of analytes' ionization took place by co-eluting endogenous substances in biological matrix. Extraction recovery rates at the spike concentrations of 500 ng/mL from all analytes were between 98% and 108%, indicating sufficient extraction efficiency.

3.4.3. Precision and stability

Assessment of precision and stability were listed in Table 5. Intra- and inter-precision (relative standard deviation, RSD) of QC samples was less than 7.0% and 8.6%, respectively. The result of stability evaluation indicated that all analytes from rat plasma were stable after culturing at an ambient temperature for 6 h and in the autosampler at 10 °C for 24 h. Therefore, the resulting data showed that the precision and repeatability of sample preparation were satisfactory for pharmacokinetic study.

3.5. Pharmacokinetic studies

From the pharmacokinetic perspective, it was considered that compounds absorbed into the blood had a chance for showing bioactivities. However, if there were some unreasonable pharmacokinetic properties, such as a very short time in the body, a constituent in the blood might not be a material basis for efficacy due to the lack of an effective blood concentration during the treatment period. Therefore, pharmacokinetic profiles of the absorbed components which were evaluated to ascertain the effective compounds *in vivo*. The validated method of RRLC-ESI-Q/TOF yielded results for the quantitation of protopine,

Table 6
Pharmacokinetic parameters of constituents after oral administration of YZP extracts.

No.	RT (min)	The analytes	C_{max} (μg/L)	T_{max} (h)	$AUC_{(0-t)}$ (h h μg/L)	Cl/F (L/h/kg)
1	10.09	Protopine	709.34 ± 146.38	4.60 ± 1.34	5570.82 ± 643.37	0.47 ± 0.05
2	11.36	Tetrahydropalmatine	858.26 ± 133.68	4.83 ± 2.14	5607.94 ± 471.05	0.86 ± 0.46
3	11.69	α-Allocryptopine	232.18 ± 74.22	3.40 ± 0.55	1167.12 ± 266.13	3.11 ± 1.21
4	13.59	Tetrahydroberberine	241.92 ± 58.63	3.83 ± 1.94	1132.88 ± 277.16	1.01 ± 0.58
5	13.71	Corydaline	855.31 ± 172.94	3.67 ± 1.21	5861.09 ± 886.95	1.48 ± 0.85
6	16.14	Byakangelicin	206.54 ± 134.27	2.83 ± 0.75	655.41 ± 327.42	1.99 ± 1.16

Values are expressed as mean ± standard deviation. F: bioavailability.

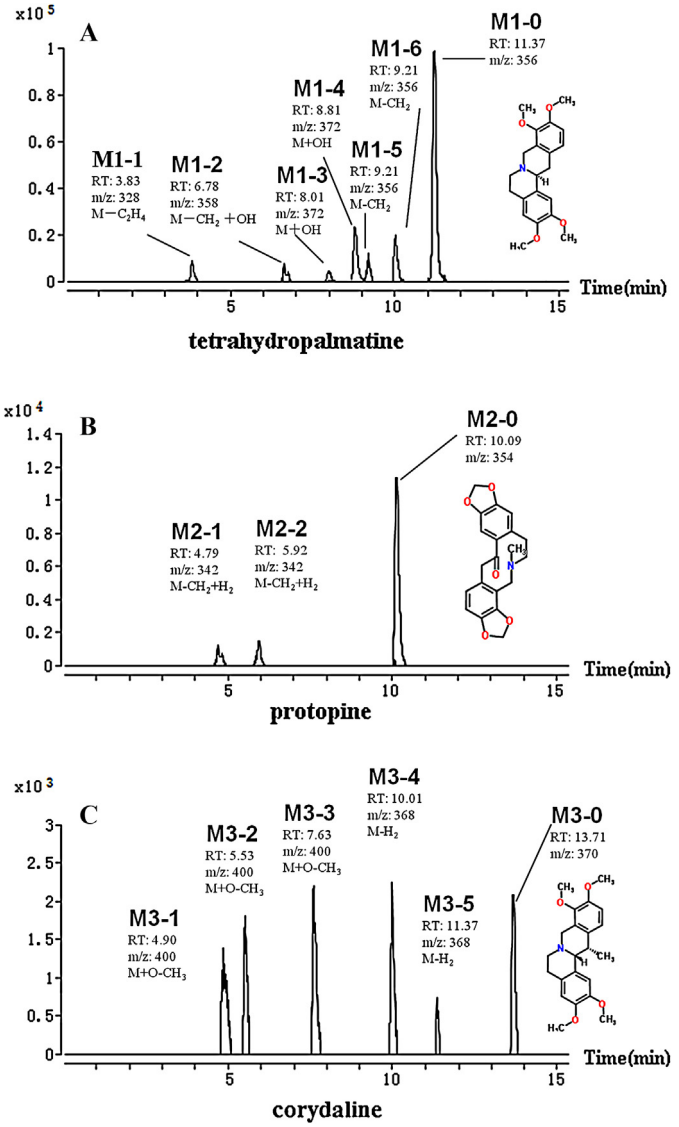

Fig. 3. The profiles of metabolites of (A) tetrahydropalmatine (M1-0), (B) protopine (M2-0) and (C) corydaline (M3-0) in rat plasma after orally administered protopine, tetrahydropalmatine and corydaline, at a dose of 1.0 mg/kg using Metabolite ID software, individually.

α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin in the rat plasma and has been applied to the pharmacokinetic study after the oral administration of YZP extracts in the rats. The mean plasma concentration *versus* time curve was presented in Fig. 4. The results suggested that the plasma concentrations of protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin in the rat plasma were lower than LLOQ after 12, 8, 12, 12, 8 and 6 h, respectively. Thus, more sensitive methods should be developed to study the pharmacokinetics of YZP systemically in the future. However, to our satisfaction, some main pharmacokinetic parameters were obtained by non-compartment model based on statistical moment such as T_{max}, C_{max} and $AUC_{(0-t)}$ et al. and showed in Table 6. The results indicated that all analytes peaked between 3.5 and 5.0 h and C_{max} ranged from 214.6 to 858.3. The information described above might be helpful for further pharmacokinetic studies on YZP.

4. Conclusion

The RRLC-ESI-Q/TOF system was proved to be a rapid and efficient for research the metabolic profiles of TCM formulation systemically. In rat plasma, twenty-one prototype constituents were detected in blood and fifteen components were identified by comparing with the chromatographic-mass spectrometric analysis of standard compounds. Moreover, seventeen prototype constituents were traced in rat CSF. Meanwhile, thirteen metabolites in the blood were obtained and concluded by Metabolite ID software after he administration of single component including protopine, tetrahydropalmatine and corydaline and TCM formulations, respectively. Importantly, a RRLC-ESI-Q/TOF method was established and validated for the simultaneous quantification of protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin in rat plasma and applied for their pharmacokinetic research. These works could provide more in-depth insights into the active components working *in vivo* and would be helpful for further revealing the pharmacology and mechanism of YZP. However, since species difference usually existed in the metabolism between the rat and human, further metabolic research in human would be needed to carry out for better understanding of this formulation.

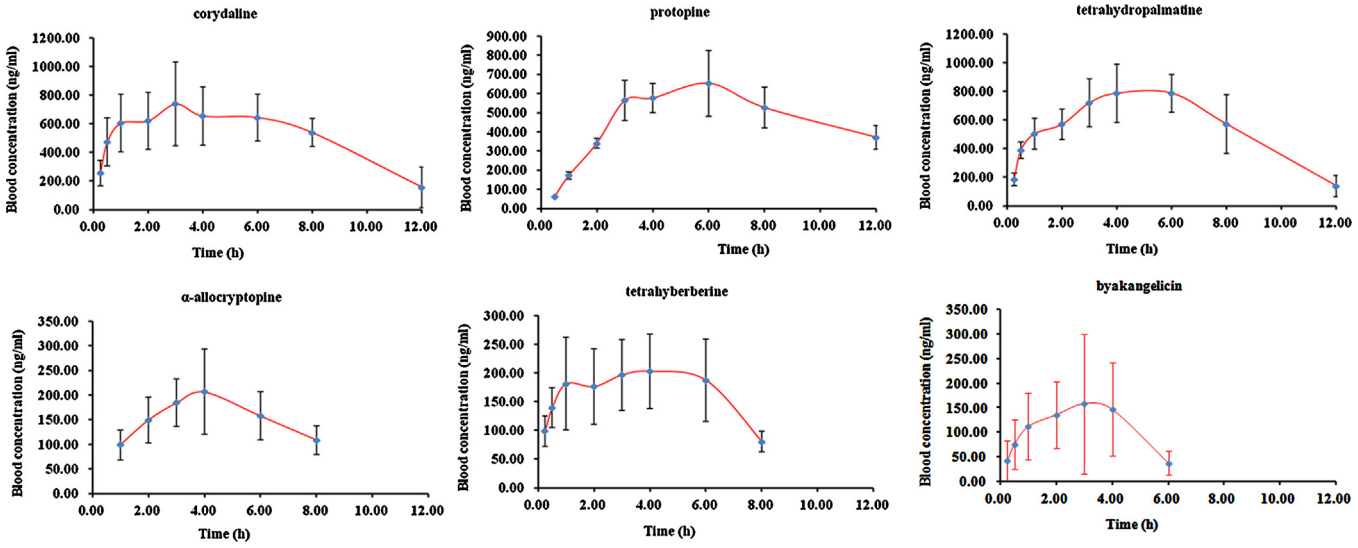

Fig. 4. The mean plasma concentration *versus* time curve of protopine, α-allocryptopine, tetrahydropalmatine, corydaline, tetrahyberberine and byakangelicin after oral administration of YZP in rats. Each point represents an average of six determinations and the error bars are standard deviations of the mean ($n = 6$).

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